Development of Dominant Rice Blast *Pi-ta* Resistance Gene Markers

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ABSTRACT

Incorporation of resistance genes into existing rice (*Oryza sativa* L.) cultivars is a powerful strategy and is commonly applied in breeding rice resistance to blast disease [caused by *Pyricularia grisea* Sacc. = *P. oryzae* Cavara (teleomorph: *Magnaporthe grisea* (Hebert) Barr)]. The rice blast resistance gene, *Pi-ta*, originally introgressed into japonica from indica rice is important in breeding for rice blast resistance worldwide. In the southern USA, the rice cultivar Katy contains *Pi-ta* and is resistant to the predominant blast *M. grisea* races IB-49 and IC-17 and has been used as the blast resistant breeding parent. Three pairs of DNA primers specific to the dominant indica *Pi-ta* gene were designed to amplify the *Pi-ta* DNA fragments by polymerase chain reaction (PCR). PCR products amplified by these *Pi-ta* specific primers were cloned and sequenced. Sequence analysis confirmed the presence of the dominant indica *Pi-ta* allele. These *Pi-ta* primers were used to examine the presence of *Pi-ta* alleles in advanced Arkansas rice breeding lines. The *Pi-ta* containing rice lines, as determined by PCR analysis, were resistant to both IB-49 and IC-17 in standard pathogenicity assays. In contrast, lines lacking the *Pi-ta* genes failed to protect rice plants against both races IB-49 and IC-17. The presence of *Pi-ta* markers correlated with the *Pi-ta* resistance spectrum. Thus, the *Pi-ta* gene markers provide a basis for stacking other blast resistance genes into high yielding and good quality advanced breeding rice lines.

Rice blast disease caused by the fungus is one of the most devastating diseases worldwide (Zeigler et al., 1994). Resistance to the pathogen is a classic gene-for-gene system, where a major resistance gene is effective against *M. grisea* strains containing the corresponding avirulence gene (Silue et al., 1992). Twenty resistance genes have been identified by extensive genetic studies (Chao et al., 1999; Mackill and Bonman, 1992; Yu et al., 1996). *Pi-b* and *Pi-ta*, two major resistance genes, introgressed from indica cultivars, have recently been molecularly characterized (Bryan et al., 2000; Inukai et al., 1994; Wang et al., 1999). Both *Pi-b* and *Pi-ta* encode predicted nucleotide binding site type proteins that are characteristics of products of major resistance genes (Wang et al., 1999; Bryan et al., 2000; Wise, 2000). Intriguingly, a single amino acid difference between resistant and susceptible indica alleles of *pi-ta* was identified from an indica cultivar C101A51 (Bryan et al., 2000). Further DNA sequence analysis of a japonica susceptible *pi-ta* allele revealed unusually low DNA polymorphism to indica resistant *Pi-ta* allele (Bryan et al., 2000; Jia et al., 2001b).

Molecular markers linked to resistance genes have been used for selection at the early seedling stages, and genotypes can be easily identified (Huang et al., 1997; Hittalmani et al., 2000). A PCR-based *Pi-ta* gene marker is useful in marker-assisted selection breeding since it is the part of resistance gene, and is simple, rapid and inexpensive and can be used for analyzing large numbers of samples. The *Pi-ta* gene marker is important for rice breeding program worldwide (Bryan et al., 2000; Hittalmani et al., 2000; Inukai et al., 1994). In the southern USA breeding programs, Katy, a japonica rice cultivar containing a tightly linked cluster of at least seven resistance genes near the *Pi-ta* locus, has been used as a blast resistant parent (Chao et al., 1999; Moldenhauer et al., 1990). Resulting progenies, such as 'Drew' and 'Kaybonnet', have been successfully released as U.S. blast-resistant cultivars (Gravois et al., 1995; Moldenhauer et al., 1998). More breeding lines based on Katy, Drew, and Kaybonnet as parents are still in the early trials (K. Moldenhauer and J. Gibbons, per. commun.).

The objectives of this research were to develop *Pi-ta* gene-specific primers to distinguish the dominant indica *Pi-ta* allele from the japonica *pi-ta* allele, to examine the presence of *Pi-ta* in 10 advanced Arkansas rice breeding lines, and to determine disease reactions of these lines to confirm the reliability of the *Pi-ta* gene marker.

MATERIALS AND METHODS

Plant Materials and Growth

Rice cultivars Katy (Moldenhauer et al., 1990), Drew (Moldenhauer et al., 1998), Kaybonnet (Gravois et al., 1995), 'Nipponbare', 'M-202' (Johnson et al., 1986), and 10 advanced rice breeding lines (experimental seeds) used in this study (Table 1) were provided by Karen Moldenhauer and James Gibbons. Seed was pregerminated on moistened filter paper for 3 d at 30°C. Seedlings were transplanted to 12.5-cm pots with a media mixture of one part sterilized local soil to one part RediEarth potting mix (Hummert, Earth City, MO). RediEarth potting is a porous lightweight, essentially sterile growing medium. It contains Vermiculite and Canadian sphagnum peat moss, the soil conditioners that help retain moisture and add aeration for plant roots. Plants were grown in a greenhouse 24 to 30°C with 16 h light for 2 to 4 wk until plants were at the 4-leaf stage for disease reaction testing and for DNA preparation.

DNA Isolation

Rice leaves were rapidly frozen in liquid nitrogen and stored at −80°C. Rice genomic DNAs were prepared from frozen leaves using DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Rice leaves were ground in liquid nitrogen to a fine powder using a mortar and pestle. DNAs were extracted using manufacturer's solutions and purified by DNeasy columns.

PCR Amplification

Primers (Table 2) synthesized by Operon Technologies, Inc. (Alameda, CA) were used for the amplification of the

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Table 1. The presence and absence of the *Pi-ta* genes in named cultivars and selected advanced Arkansas rice breeding lines.

Cultivars/lines	Parent†	Presence‡	Disease reaction
Katy	Tetep	*Pi-ta*	R¶
Drew	Katy	*Pi-ta*	R
Kaybonnet	Katy	*Pi-ta*	R
Nipponbare	N/A§	N	S
M-202	N/A	No	S
117	Katy	No	S
209	Katy	No	S
406	Katy	*Pi-ta*	R
602	Katy	*Pi-ta*	R
605	Katy	*Pi-ta*	R
606	Katy	*Pi-ta*	R
1142	Katy	No	S
1153	Katy	No	S
1203	Kaybonnet	*Pi-ta*	R
1204	Kaybonnet	No	S

† Parent used for production of advanced breeding lines.
‡ Indicates the presence of the *Pi-ta* gene as determined by three sets of primers (YL153/YL154, YL155/YL87 and YL100/YL102).
§ N/A indicates not applicable.
¶ R indicates resistant; S indicates susceptible (disease reactions were determined using both IB-49 and IC-17 races of *M. grisea*).

genomic DNA from the rice cultivars and advanced breeding lines by PCR. Each PCR reaction consisted of 10 to 20 ng of total DNA, 10 mL of Taq PCR Master Mix [(2× concentrated) containing 0.5 unit Taq DNA Polymerase, Qiagen PCR Buffer (with 3 mmol $MgCl_2$)] and 400 mmol of each dNTP (Taq PCR Master Mix Kit, Qiagen), 2 μL of $MgCl_2$ (25 mmol), 1mL of primer 1 (10 mmol), and primer 2 (10 mmol) each in a final volume of 20 mL. The PCR reactions were performed in a Peltier Thermal Cycler (PTC-20, MJ Research, Waltham, MA) with the following program: 3 min at 95°C for initial denaturation followed by 29 cycles of 30 s at 95°C, 30 s at 55°C for YL 153/YL154 and YL155/YL87 and at 64.5°C for YL100/YL102, 30 s each at 72°C; and final extension at 72°C for 7 min. Aliquots (10 μL) of each of the PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels in 1× TBE buffer, then stained in ethidium bromide and visualized by means of an ultraviolet transilluminator.

Cloning and Sequence of PCR Products

PCR products amplified from rice cultivars Katy, Kaybonnet, and Drew were cloned into pDrive Cloning Vector modified on the basis of the manufacturer's recommendation (Qiagen). Briefly, 1 μL pDrive vector was mixed with 1 to 4 μL of the PCR product and 5 μL of the ligation master mix for 1 h at 4°C and transformed by electroporation. Resulting plasmids were identified by blue-white selection and plasmid DNA was purified by means of Qiagen Plasmid Mini Preparation Kits. The sequences of inserts were determined by means of ABI-PRISM BigDye Terminator Cycle Sequencing on ABI 377 (Applied Biosystems, Foster City, CA). Sequence analysis was performed with Vector NTI suite (InforMax).

Disease Reactions

Magnaporthe grisea race IB-49 (ZN52) and IC-17 (ZN57) (Correll et al., 2000) were provided by Fleet N. Lee (Professor of Plant Pathology, University of Arkansas, Rice Research and Extension Center). Disease reactions of breeding lines were performed with standard pathogenicity assays (Valent et al., 1991). Briefly, plants were grown in a greenhouse, to the three to four-leaf stage, and then inoculated with 2 mL of spore suspensions (2.5×10^5 spores/mL) with an airbrush. Plants were inoculated inside a plastic bag that was then sealed to maintain high humidity. After 24 h, the plants were removed from the bags and returned to the greenhouse. Disease reactions were determined 7 d after inoculation. Resistant reaction was based on no visible infection and no conidia produced from affected tissue. Susceptible reaction was based on a lesion size greater than 3 cm in length, visible infection, and conidia evident in affected tissue (Valent, 1997). The resistance and susceptibility of each cultivar and line were determined on the basis of obtaining the same disease reactions using three plants in each pot with three repeats.

RESULTS

Development of the Indica *Pi-ta* Gene Markers

To develop a *Pi-ta* gene specific primer, the nucleotide that distinguishes the dominant indica *Pi-ta* allele from susceptible japonica *pi-ta* alleles was included as the last base (Jia et al., 2001b). Two pairs of primers, YL153/YL154 and YL100/YL102 (Table 2, Fig. 1), were designed to amplify specifically regions of the *Pi-ta* gene encoding the translation start site (YL153/YL154) and the carboxyl terminus (YL100/YL102). The specificity of PCR reaction can be affected by the annealing temperature. The optimal annealing temperature for each set of primers is listed in Table 2. *Pi-ta* containing rice cultivars Katy and Drew (Jia et al., 2001b) were used as positive controls. Rice cultivar Nipponbare that does not contain *Pi-ta* (Bryan et al., 2000) was used as the negative control. As shown in Fig. 1B, DNA fragments of 403 (YL100/YL102) and 440 base pairs (bp) (YL153/YL154) were amplified from Katy and Drew, respectively, and the same size fragments were also amplified from Kaybonnet suggesting that *Pi-ta* is also present in Kaybonnet. The absence of PCR product from M-202 indicates that it does not contain *Pi-ta*. These results

Table 2. Sequences of 19- or 20-mer oligonucleotide primers for the dominant *Pi-ta* specific markers and optimized annealing temperatures.

Locus†	Primer	Sequence (5′–3′) (Number of bp)‡	Annealing temperature (°C)	Location of primer§
$Pi\text{-}ta_{440}$	YL153	CAACAATTTAATCATACACG$_{(20)}$	55	2021-2040
	YL154	ATGACACCCTGCGATGCAA$_{(19)}$		2460-2442
$Pi\text{-}ta_{1042}$	YL155	AGCAGGTTATAAGCTAGGCC$_{(20)}$	55	4409-4428
	YL87	CTACCAACAAGTTCATCAAA$_{(20)}$		5450-5431
$Pi\text{-}ta_{403}$	YL100	CAATGCCGAGTGTGCAAAGG$_{(20)}$	64.5	6257-6276
	YL102	TCAGGTTGAAGATGCATAGC$_{(20)}$		6659-6640

† The subscript number refers to the size in base paris (bp) of the amplified dominant product.
‡ Polymorphic nucleotides were underlined.
§ Location of the *Pi-ta* gene was based on GenBank accession no. AF207842.

suggest that both primer pairs were specific for the dominant indica *Pi-ta* allele.

To verify the presence of the entire *Pi-ta* gene, DNA primers YL155/YL87 specific to the middle region were designed for the PCR amplification. Consistent with results obtained by primers YL100/YL102 and YL153/YL154, a predicted fragment of 1042 bp was also specifically amplified from Katy, Drew, and Kaybonnet. Similarly, no amplification was obtained from Nipponbare or M-202 (Fig. 1B).

To verify the amplification of the dominant indica *Pi-ta* allele, PCR products were cloned into pDrive vector, and sequenced. Searching the current GenBank database (http://www.ncbi.nlm.nih.gov/blast; verified May 22, 2002) for sequences of all PCR products revealed 100% identity with DNA fragments from nucleotide positions 2021-2442 (YL153 and YL154), 4409-5450 (YL155 and YL87), and 6257 to 6659 (YL100 and YL102) of the *Pi-ta* gene (Table 2, GenBank accession no. AF207842). These results indicate that portions of the indica dominant *Pi-ta* alleles were amplified from Katy, Drew, and Kaybonnet.

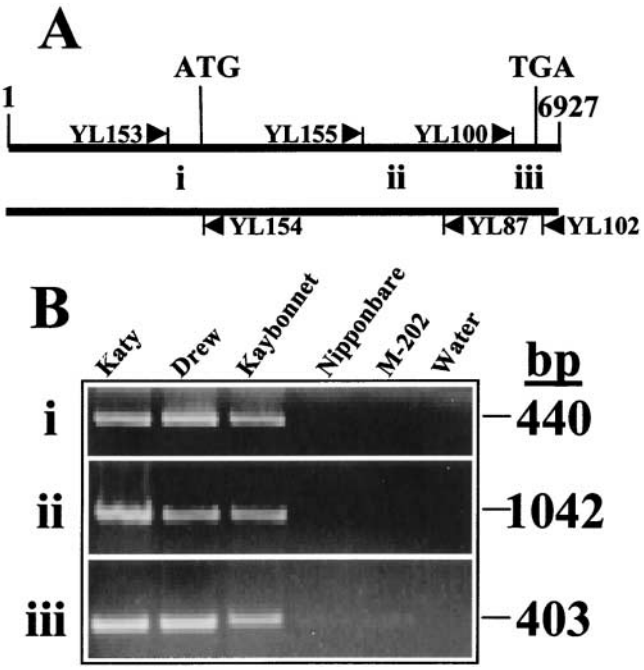

Fig. 1. A schematic representation of the double strands of the 6927 bp of the *Pi-ta* gene (A) and PCR products from genomic DNA prepared from Katy (1), Drew (2), Kaybonnet (3), Nipponbare (4), and M-202 (5) amplified by the dominant *Pi-ta*-specific primers. Line 6 is the water control (B). Names and locations of primers and region amplified by PCR using primers YL153 and YL154 (i), YL155/YL87 (ii), and YL100/YL102 (iii) are shown in A. The sizes of fragments were estimated by mean of kilobase markers. ATG indicates the translation start site and TGA indicates the termination site.

Detecting the Presence of *Pi-ta* in Advanced Arkansas Breeding Lines by PCR

To demonstrate the utilization of *Pi-ta* gene markers in breeding, 10 advanced breeding lines based on Katy, Drew, and Kaybonnet as parents were used for PCR amplification. Katy, Drew, and Kaybonnet were used as positive controls and Nipponbare and M-202 were used as negative controls. The presence and absence of the *Pi-ta* genes were all consistent with all three dominant primer pairs (YL153/YL154, YL155/YL87, and YL100/YL102). As shown in Table 1, five of 10 breeding lines contain the *Pi-ta* gene and the other lines do not contain the *Pi-ta* gene.

Disease Reactions to Predominant Arkansas *M. grisea* Races IB-49 and IC-17

Katy, Drew, and Kaybonnet, which are resistant to IB-49 and IC-17 (Jia et al., 2001a; Moldenhauer et al., 1992), were used as positive controls. M-202, which is susceptible to IB-49 and IC-17, was used as a negative control (Jia et al., 2001a). The controls, Nipponbare, and advanced breeding lines were inoculated with avirulent *M. grisea* races IB-49 and IC-17 to confirm the spectrum of the *Pi-ta* resistance. As shown in Table 1, Nipponbare, lacking *Pi-ta*, was susceptible to both IB-49 and IC-17. All *Pi-ta* containing lines were resistant to both IB-49 and IC-17 (Table 1). On the other hand, those rice lines that do not contain *Pi-ta*, as determined by PCR analysis, were susceptible to IB-49 and IC-17 (Table 1). Thus, the presence of the *Pi-ta* marker correlated well with the spectrum of *Pi-ta* resistance.

DISCUSSION

Two major challenges to rice breeders are the selection of appropriate resistance genes and prediction of the stability of resistance in rice cultivars with such gene combinations. A simple but efficient method to analyze blast resistance genes is necessary for rice breeding programs where many cultivars with many resistance genes are used for crossing and many breeding lines are maintained. The genotypes of major genes for blast resistance can be deduced through their reaction patterns following inoculations with blast isolates that attack known resistance genes. Rice blast follows a gene-for-gene recognition where a major resistance gene specifically interacts with the corresponding fungal avirulence gene (Silue et al., 1992). The instability of avirulence genes in the rice blast fungus (Valent, 1997) and overlapping spectra of race-specific resistance are major limiting factors. For rapid development of resistant cultivars, resistance-gene-mediated selection for gene stacking is one of the more reliable approaches. Cloning resistance genes have made it possible to develop resistance gene markers.

We have developed optimal condition for utilizing a dominant *Pi-ta* gene marker. DNA polymorphisms of nucleotides between resistant indica *Pi-ta* allele and susceptible japonica *pi-ta* alleles (Jia et al., 2001b) allowed us to develop the *Pi-ta* gene markers. DNA primers were designed to perform in PCR conditions as defined above (Table 2), and only the resistant indica *Pi-ta* allele were amplified. Only two nucleotides in each pair of the primers (YL100/YL102 and YL153/YL154) distinguish

them from the recessive japonica *pi-ta* allele. Resulting PCR products distinguish the dominant indica *Pi-ta* allele from the recessive japonica *pi-ta* allele. Precautions were taken throughout this work to avoid the introduction of contaminating DNA. High quality DNA polymerase, $MgCl_2$ concentration, and annealing temperatures for each primer set were all critical factors that contributed to the successful determination of *Pi-ta* gene presence. Primers that have been thawed repeatedly are not recommended for the amplification. Primers YL100/YL102 distinguishing dominant indica *Pi-ta* allele with a single nucleotide from recessive indica *pi-ta* allele (Bryan et al., 2000) amplify both dominant and recessive indica *Pi-ta* alleles (Wang and Jia, unpublished data). Determination of the dominant *Pi-ta* alleles in indica breeding lines still awaits the sequence confirmation of PCR products amplified byYL100/YL102 primers.

In the southern USA, blast resistance provided by Katy has lasted over the decade since its release. Katy contains a cluster of resistance genes at the *Pi-ta* region near the centromere of chromosome 12 (Bryan et al., 2000; Chao et al., 1999; Moldenhauer et al., 1992). Decreased recombination near the centromere (Bryan et al., 2000) may facilitate the incorporation of tightly linked resistance genes into new breeding lines by means of these *Pi-ta* gene markers. Thus, the *Pi-ta* gene markers may serve as an indicator for a cluster of resistance genes. Selection of breeding parents for a broader-spectrum blast resistance may be achieved with these *Pi-ta* gene markers.

Using *Pi-ta* gene specific PCR not only assists plant breeders in making parental selection but also can facilitate stacking resistance genes into advanced breeding lines. If advanced breeding lines occur that are resistant to IB-49 and IC-17 but do not contain *Pi-ta*, then these lines may contain other resistance genes against IB-49 and IC-17. Subsequent incorporation of *Pi-ta* and other resistance genes will lead to broader spectra of the resistance. However, if advanced breeding lines occur that are resistant to IB-49 and IC-17 and contain *Pi-ta*, the presence of other blast resistance genes can be determined by virulent fungal isolates that infect *Pi-ta* containing lines. Subsequent resistant reactions indicate that these lines also contain other blast resistance genes. Thus, *Pi-ta* is stacked with other resistance genes into advanced breeding lines. Alternatively, selection for other resistance genes can be achieved through marker-assisted breeding (Huang et al., 1997; Hittalmani et al., 2000).

In this study, we demonstrated the development of the first dominant resistance gene markers using the DNA sequence of the cloned gene in the rice blast system. Increasing efforts to clone more resistance genes (Wang et al., 1999; Wise, 2000) worldwide will accelerate the development of more dominant resistance gene markers for molecular breeding, thereby accelerating introduction of durable, broad-spectrum disease resistance into high yield, good quality rice cultivars.

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